Regulatory responses of Streptococcus pneumoniae to varying metal ion- and nitrogen availability

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Chapter 2

To Have Neighbour's Fare: Extending the Molecular Toolbox for *Streptococcus pneumoniae*

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Abstract

In past years, several useful genetic tools have been developed to study the molecular biology of *Streptococcus pneumoniae*. In order to extend the existing spectrum of tools, advantage was taken of the toolbox originally developed for the closely related bacterium *Lactococcus lactis*, which was adapted for the manipulation of *S. pneumoniae*. The modified tools are as follows: (i) An improved nisin-inducible (over)expression system (NICE). The *nisRK* genes, encoding a two-component system essential for transcriptional activation in response to nisin, were integrated into the *bgaA* locus of *S. pneumoniae* D39. In this strain, D39*nisRK*, addition of nisin resulted in the overexpression of several genes placed under control of the nisin-inducible promoter, while no detectable expression was observed in the absence of nisin. (ii) A *lacZ* reporter system. Using strain D39*nisRK*, which lacks endogenous β-galactosidase activity, the usefulness of the *lacZ* reporter vector pORI13 for the generation of chromosomal transcriptional fusions was demonstrated. In addition, the *repA* gene, necessary for the replication of pORI13, was introduced into the *bgaA* locus, thereby generating a background for plasmid-based promoter expression studies. (iii) A simplified chemically defined medium, which supports growth of all sequenced *S. pneumoniae* strains to a level comparable to that in complex medium. (iv) A system for the introduction of unmarked deletions and mutations into the chromosome, which is independent of the genotype of the target strain. Most of these systems were successfully applied in strains R6 and TIGR4 as well. In addition, the tools offer several improvements and advantages compared to existing ones. Thus, the molecular toolbox for *S. pneumoniae* has been successfully extended.

Introduction

Streptococcus pneumoniae is a human pathogen, and causes meningitis, pneumonia, otitis media and bacteraemia, especially in children and the elderly. Treatment and prevention of infection is primarily based on antibiotics and vaccines (40). Current vaccines are directed against the polysaccharide capsule, but they do not cover the entire spectrum of different serotypes and may offer only limited protection in children younger than 2 years (40). Furthermore, multi-antibiotic-resistant strains of *S. pneumoniae* have emerged in recent years, and are now a serious problem (115). To be able to combat infections caused by *S. pneumoniae* in the future, new vaccines and antimicrobials are required. Therefore, a major effort is currently being made to dissect the molecular mechanisms underlying infection by *S. pneumoniae*.

Various molecular tools have been described to study the molecular biology of *S. pneumoniae*, such as inducible promoters, *lacZ* and green fluorescent protein (GFP) reporter vectors, and methods to construct mutant strains (1,56,62,86,185,310).

Here, we report on the development and use of several additional molecular tools for *S. pneumoniae*, which were adapted from existing tools for the related bacterium *Lactococcus lactis*. These include an improved nisin-controlled gene expression system (NICE), a chemically defined medium (CDM), a plasmid-based *lacZ* reporter system, and a method to generate unmarked mutations in the chromosome. These tools extend the existing toolkit for the basic genetic manipulation of *S. pneumoniae*, and they are applicable in the sequenced strains R6 (D39) and TIGR4, and, most likely, in other strains as well.

Materials and Methods

Strains and growth conditions

Strains used in this study are listed in Table 1 and were stored in 10% glycerol (v/v) at -80ºC. *S. pneumoniae* was routinely grown as standing cultures in M17 broth (316) containing 0.25% (w/v) glucose (GM17) or in Todd–Hewitt broth supplemented with 0,5% yeast extract (THY) at 37°C. For growth on plates 1-5% (v/v) defibrinated sheep-blood (Johnny Rottier, Kloosterzande, The Netherlands) was added to GM17 agar. *L. lactis* was grown as standing cultures in M17 supplemented with 0.5% (w/v) glucose at 30°C. *E. coli* EC1000 was grown in TY broth in a shaking incubator at 37°C. Chloramphenicol and erythromycin were used in concentrations of 2.5 and 0.25 μg/ml for *S. pneumoniae* and 5 and 4 μg/ml for *L. lactis*, respectively. Erythromycin was used in a concentration of 120 μg/ml for *E. coli*. Trimethoprim was used in a concentration of 15 μg/ml for *S. pneumoniae*. When appropriate, 0.006% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was used in plates. A nisin stock was obtained by extracting nisaplin (Aplin and Barrett, Danisco, Denmark) 1:1 (w/v) with 50% ethanol, spinning the suspension for 1 min at 20,000 g in a table top centrifuge and discarding the pellet. The supernatant contains approximately 20 mg nisin per ml and was stored at -20ºC. For induction of the *nisA* promoter, nisin was used in a concentration as indicated in the Results section. Working stocks with the appropriate nisin concentration were freshly prepared in 50% ethanol before each experiment.

DNA isolation and manipulation

Chromosomal DNA was isolated from *L. lactis* and *S. pneumoniae* according to the method of Johansen and Kibenich (144). Plasmid DNA was isolated using the plasmid isolation kit from Roche. DNA manipulation was done according to standard procedures. *L. lactis* and *E. coli* were transformed with plasmid DNA by electroporation (132). *S. pneumoniae* was transformed by growing the strain at 37°C in GM17 to an OD_{600} of 0.1. Competence-stimulating peptide CSP-1 was used at 100 ng/ml to induce competence (256). Transformants were selected on GM17 agar with the appropriate antibiotic. Southern blotting was done as described (60). Probe labeling, hybridization and detection were performed using the ECL direct nucleic acid labeling system according to the specifications of the manufacturer (Amersham Pharmacia Biotech).

Plasmid and strain construction

Primers used in this study are listed in Table 2. The *nisRK* genes were introduced into the *bgaA* gene (*spr0565*) on the chromosome of *S. pneumoniae* as follows: Primers trpm-Fp and trmp-Rp were used to PCR amplify the *trmpR* marker from pKOT. Using *E. coli* EC1000 as a host, the PCR fragment was inserted into the *Hind*III site of pORI28 in the same orientation as the *emR* gene, yielding pTK1. The 5' and 3' ends of *bgaA* gene were amplified from D39 chromosomal DNA with primer pairs bgaA-1/bgaA-2 and bgaA-3/bgaA-4 and were cloned into the *Mlu*I/*Bam*HI and *Kpn*I/*Bgl*II sites of pTK1, respectively, resulting in pTK2. Finally, the *nisRK* genes, which were amplified from chromosomal DNA of *L. lactis* NZ9700 with primers NisRK-Fp-term/NisRK-Rp, were cloned into the *Pst*I/*Bam*HI sites of pTK2 resulting in pTK3. Both pTK2 and pTK3 were generated in *L. lactis* LL108.

The *repA* gene was introduced into the *bgaA* locus in a similar manner using *E. coli* EC1000 as a host. Approximately 800 bp regions surrounding *bgaA* were amplified from chromosomal DNA of D39 using primers R6_bgaA-5/6 and R6_bgaA-7/8 and cloned into the *Xba*I/*Bam*HI and *Kpn*I/*Bgl*II sites of pTK1, resulting in pTK4. Subsequently, the *repA* gene, amplified from plasmid pUC23rep3 with primers RepA_Fp-term/RepA_Rprimer, was cloned into the *Xba*I/*Pst*I sites of pTK4, giving pTK5.

Table 1. Strains and plasmids used in this study.
Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Km^R, kanamycin resistance; Spec^R, spectinomycin resistance; Trmp^R, trimethoprim resistance.

Strain/plasmid	Description	Reference or source
S. pneumoniae		
D39	Serotype 2 strain, *cps2*	(19)
TIGR4	Serotype 4 strain	(317)
R6	D39_(*cps*2 2538–9862) with increased transformation efficiency	(133)
D39*nisRK*	D39 Δ*bgaA::nisRK*;Trmp^R	This work
D39*repA*	D39 Δ*bgaA::repA*;Trmp^R	This work
TIGR4*nisRK*	TIGR4 Δ*bgaA::nisRK*;Trmp^R	This work
TIGR4*repA*	TIGR4 Δ*bgaA::repA*;Trmp^R	This work
R6*nisRK*	R6 Δ*bgaA::nisRK*;Trmp^R	This work
R6repA	R6 Δ*bgaA::repA*;Trmp^R	This work
TK100	D39*nisRK* Δ*glnA*;Spec^R	(162)
TK101	D39 Δ*glnR*, containing unmarked chromosomal deletion of *glnR*	This work
L. lactis		
NZ9000	MG1363Δ*pepN*::*nisRK*	(171)
LL108	MG1363 RepA$^+$ (multi-copy); Cm^R	(186)
E. coli		
EC1000	Km^R; MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	(187)
Plasmids		
pORI13	Em^R; *ori*$^+$ *repA*$^-$; promoterless *lacZ*	(277)
pORI280	Em^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01; constitutive *lacZ* expression from P32 promoter	(187)
pORI28	Em^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01;	(187)
pNZ8048	Cm^R; Nisin inducible P*nisA*	(74)
pNG8048E	Cm^R Em^R; Nisin inducible P*nisA*, pNZ8048 derivative.	Laboratory collection
pKOT	Amp^R Trmp^R; pBluescript II KS+ containing *trmp* gene under constitutive P32 promoter	Laboratory collection, Hermans, P.W.
pUC23rep3	Amp^R; carrying the pWVOI *repA* gene under control of promoter P23	(189)
pJB1	Cm^R; pNG8048E carrying *spr0567-myc* behind *nisA* promoter	This work
pJB2	Em^R; pORI13::P*cpsA*	This work
pTK1	Em^R Trmp^R; pORI28 containing P32::*trmp* gene from pKOT	This work
pTK2	Em^R Trmp^R; pTK1 containing left and right parts of D39 *bgaA*	This work
pTK3	Em^R Trmp^R; pTK2 containing NZ9700 *nisRK* genes	This work
pTK4	Em^R Trmp^R; pTK1 containing left and right flanking regions from D39 *bgaA*	This work
pTK5	Em^R Trmp^R; pTK4 containing *repA* from pUC23rep3	This work
pTK6	Cm^R; pNG8048E carrying *glnA* behind *nisA* promoter	This work
pTK7	Em^R; pORI280 carrying *glnR* deletion	This work

Table 2. Oligonucleotide primers used in this study. Stem-loop structure (predicted ΔG° -20.8 kcal mole^{-1}) in bold. Restriction enzyme sites underlined.

Name	Nucleotide sequence (5' to 3')	Restriction site
trmp-Fp	CCCCAAGCTTGGATTTTTGTGAGCTTGGA	*Hind*III
trmp-Rp	CCCCAAGCTTGTTACGACGCGCATAGACGG	*Hind*III
bgaA-1	GCTCTAGACAAATCGTTGAACGAGGTGG	*Xba*I
bgaA-2	CGGGATCCCCTGTCCAGATAAACTGTCC	*Bam*HI
bgaA-3	GGGGTACCCGCTACCGTCGTTCCAAGCG	*Kpn*I

bgaA-4	GAAGATCTGTAATTTGATAGTCTTGACG	*Bgl*II
nisRK-Fp-term*	CGGGATCC**AGGGAAGGGAGGTGAGAGCCTCCCTTCCCT AAAAAA**GTGTGTATCTCAATCCTTGG	*Bam*HI
nisRK-Rp	AAACTGCAGAATCCTTAGAGATTAC	*Pst*I
R6_bgaA-5	CGGGATCCCCAAGTGGTATACATGGTATGAC	*Bam*HI
R6_bgaA-6	GCTCTAGAGTTGCTACTAAACGTCTTCAAGG	*Xba*I
R6_bgaA-7	GGGGTACCGCAGGATTAGTAGTTACTAAAGG	*Kpn*I
R6_bgaA-8	GAAGATCTGAAAACGACAGAGAAATTCTCC	*Bgl*II
RepA-Fprimer_term	CGGGATCC**AGGGAAGGGAGGTGAGAGCCTCCCTTCCCT AAAAAA**TTCGAGCTCGCCCATCCTTTCAT	*Bam*HI
RepA-Rprimer	AAAACTGCAGCCCCTTCGACTTTCGTCAGGGGGC	*Pst*I
glnA_R6-7	GGCGGTCTCTCATGCCAATCACAGCTGCAG	*Bsa*I
glnA_R6-8	GCTCTAGATGTGACTGTTTACCCACAG	*Xba*I
659for	CATGCCATGGAAAAATGGCAAACATGTG	*Nco*I
659rev	TGCTCTAGACTAGTTAAGATCTTCTTCTGAAATAAGTTTTT GTTCGGCTAATTCCTTCAAAGTTTG	*Xba*I
cpsArev+50	AACTGCAGACACTTCGCTTCACTTTCTG	*Pst*I
cpsAfor-400	CGGGATCCAAGTACCGCCATAGAGCG	*Xma*I
glnR_R6-1	TGCTCTAGAGGTGGTGACTCAGCTGCCGC	*Xba*I
glnR_R6-2	CGGGATCCCCTTCATTTACAATTTCCTTC	*Bam*HI
glnR-R6-3-ncoi	CATGCCATGGGGTCGCGGTTAGGCAACCGC	*Nco*I
glnR_R6-4	GAAGATCTCCTTCTGCATCAAACAAGG	*Bgl*II

S. pneumoniae D39*nisRK*, harbouring the *nisRK* genes integrated via double cross-over in, the *bgaA* locus was constructed by transformation with a PCR product obtained with pTK3 as a template using primers bgaA-1/bgaA-4 and selecting for trimethoprim resistant clones. In analogy, a gene cassette was obtained from pTK5 by PCR with primers R6_bgaA-6/R6_bgaA-8, which was used to construct *S. pneumoniae* D39*repA*. Correctness of the mutations was verified by PCR and Southern blotting. In the same way strains TIGR4*nisRK*, TIGR4*repA*, R6*nisRK* and R6*repA* were constructed.

Plasmids pTK6 and pJB1 containing *glnA* (*spr0444*) and *spr0576* under control of the nisin-inducible *nisA* promoter were constructed by amplifying the respective genes from chromosomal DNA of strain D39 using primers pairs glnA_R6-7/glnA_R6-8 and 659for/659rev and cloning the resulting amplicons into the *Nco*I/*Xba*I sites of pNG8048E. The *spr576* gene was fused to a C-terminal Myc-tag. *L. lactis* NZ9000 was used as the cloning host.

To construct a transcriptional *lacZ* fusion to the promoter of *cpsA*, the fragment was PCR amplified from chromosomal DNA of *S. pneumoniae* D39 with primer pair cpsArev+50/cpsAfor-400 and cloned into the *Xma*I/*Pst*I sites of pORI13, yielding pJB2. *L. lactis* LL108 was used as the cloning host.

pTK7, containing the deleted *glnR* gene (*spr0443*) surrounded by approximately 800 bp fragments, was constructed by cloning the PCR products amplified from D39 chromosomal DNA with primer pairs glnR_R6-1/glnR_R6-2 and glnR-R6-3-ncoi/glnR_R6-4 in the *Xba*I/*BamH*I and *Nco*I/*Bgl*II sites of pORI280, using *L. lactis* LL108 as a host.

Enzyme assays

Cell-free extracts were made by resuspending cell pellets from 2 ml of culture in 20 mM Tris/HCl pH 7.5 and disruption of cells by shaking them for 1 min with glassbeads (75-150 μm) in a Biospec Mini-BeadBeater-8. Glutamine synthetase (GS) activity (transferase reaction) was determined as described by Fisher & Sonenshein (92). β-Galactosidase activity was determined in cell suspensions permeabilized by chloroform and SDS, as described by Israelsen *et al*. (137).

Detection of the Spr576-Myc fusion protein by Western blotting

Cells from 1 ml cell culture grown in GM17 were harvested in 1 ml PBS and washed twice. Whole-cell samples were diluted in sample buffer (4% sodium dodecyl sulphate, 2% 2-mercaptoethanol, 20% (v/v) glycerol, 125 mM Tris/HCl pH 6.8, 0.1 mg ml-1 bromophenol blue) heated for 5 minutes at 100°C, fractionated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. Spr576-Myc was detected with ECL (Amersham Biosciences) on immunoblots after incubation with a 1:5,000 dilution of an anti-Myc monoclonal antibody (Gentaur, Brussels, Belgium), followed by a 1:10,000 dilution of anti-mouse IgG peroxidase (Amersham Biosciences).

Results and Discussion

Construction of an improved NICE system for overexpression in *S. pneumoniae*

NICE is used mainly in *L. lactis*, but has also been applied successfully in other Gram-positive bacteria (86,86,159,214). The core of NICE is formed by the NisRK two-component system, which in the presence of nisin activates expression of the *nisA* and *nisP* promoters in a dose-dependent manner (73,169). Nisin is a peptide bacteriocin belonging to the class of lantibiotics and is produced by certain strains of *L. lactis*. An advantage of induction of expression with nisin is that it is metabolically inert at sub-inhibitory concentrations, as opposed to sugars or other metabolically active compounds that have been used in *S. pneumoniae* (1,56).

Two previous studies describe the application of NICE in *S. pneumoniae* (86,200). In the first study two plasmids were used, one containing the nisin-inducible promoter, the other the *nisRK* genes. In this situation, transcription from the nisin-inducible promoter also occurred in the absence of nisin. Furthermore, nisin concentrations just below the growth inhibitory level resulted in only a tenfold overproduction (86). The second study used a single plasmid that contains both the nisin-inducible promoter as well as the *nisRK* genes, but in this case, nisin-induced expression was observed only at 30^{o}C (200).

To circumvent these problems, we have constructed a stable derivative of *S. pneumoniae* D39 (D39*nisRK*) that harbours *nisRK* with their native promoter, into the *bgaA* locus (Fig. 1A), thereby abolishing endogenous β-galactosidase activity (Fig. 3B). To test whether NICE is functional in strain D39*nisRK*, the genes encoding glutamine synthetase GlnA and the putatively secreted protein Spr0576 were placed under the control of the *nisA* promoter in plasmid pNG8048E. In the absence of nisin, there was no detectable expression from the *nisA* promoter (Fig. 1B, C). Addition of nisin to the medium resulted in expression of *glnA*, which was dose-dependent (Fig. 1b). At nisin concentrations of 2 to 20 ng/ml, which is far below the MIC value of 500 ng/ml (168), a 20 to 50-fold higher glutamine-synthetase (GS) activity was observed compared to the wild-type (Fig. 1B). Nisin-induced overexpression of *spr0576* resulted in the appearance of two bands on the Western blot (Fig. 1C). The lower band probably represents the protein with the putative signal sequence cut off, and the upper band the unprocessed protein. The appearance of these two bands indicates that nisin induction leads to the production of large quantities of the Myc-tagged Spr0576. Furthermore, we also showed that nisin-induced expression occurs both in the early and the late exponential phase of growth (Fig. 1C).

The *nisRK* integration cassette could be easily transferred to other strains via double cross-over and selection for resistance against the rarely used antibiotic trimethoprim. In addition, nisin-dependent expression of GlnA and Spr0576 was equally well functional in TIGR4 and R6 derivatives that carry *nisRK* integrated in the *bgaA* locus (data not shown).

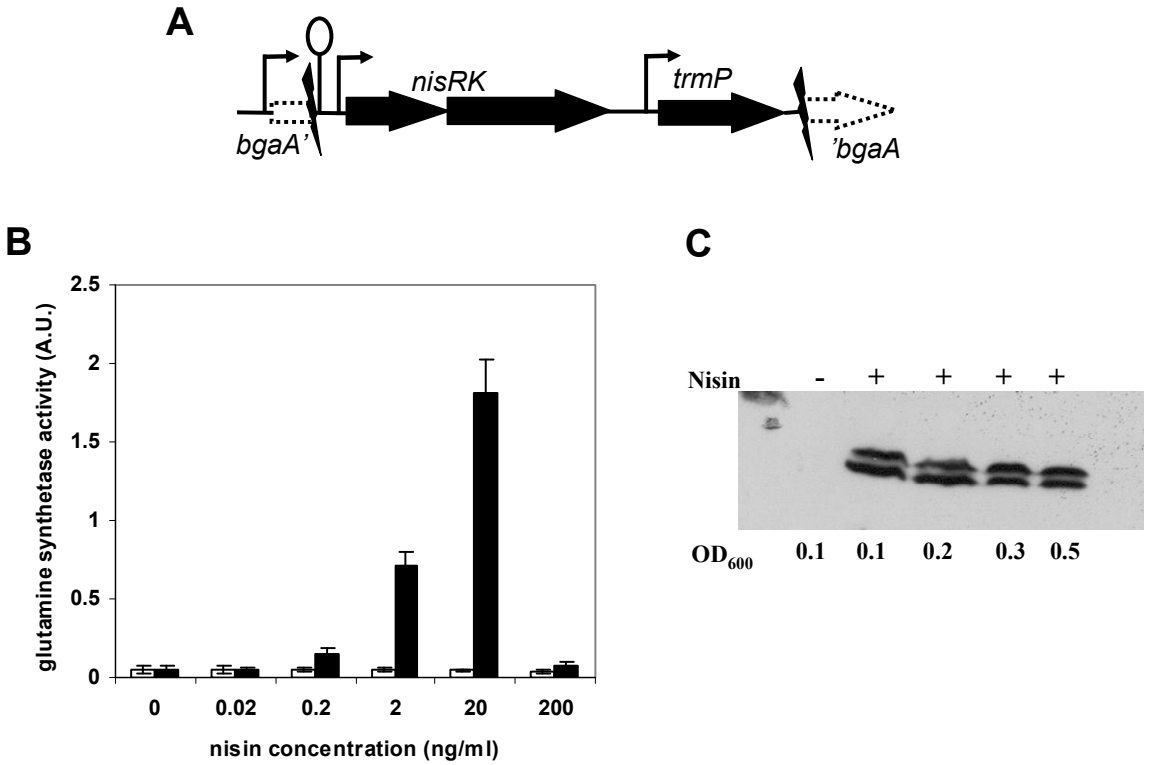

Figure 1. Characterization of the improved nisin-inducible system. (A) Schematic representation of the *nisRK* genes of *L. lactis* integrated in the *bgaA* locus on the chromosome of *S. pneumoniae*. The integration led to the removal of an approximately 3 kb internal fragment of *bgaA*. *trmp*: trimethoprim-resistance marker. Stem-loop indicates terminator structure (predicted ΔG° -20.8 kcal/mole). Right-pointing arrows are promoters. Lightning symbols represent the site of insertion of the *nisRK* fragment. The dotted arrow shows the position of the disrupted *bgaA* gene. (B) Nisin concentration-dependent expression of *glnA* in *S. pneumoniae* D39*nisRK*, harbouring *glnA* cloned behind the *nisA* promoter of pNG8048E (pTK6, black bars) or the empty plasmid pNG8048E (white bars). Induction with nisin was done for 1 hour at OD_{600} of 0.2 in GM17. (C) Western blot showing nisin-induced expression of the Myc-tagged Spr0576 in D39*nisRK* harbouring plasmid JB1 at different OD_{600} during exponential growth in GM17. The lower band represents Spr0576 with the signal sequence cut off, and the upper band the unprocessed protein. Induction (+) was done for 1 hour with 2 ng/ml nisin. -, No addition of nisin.

Although THY and Brain Heart Infusion (BHI) are often used as a growth media for *S. pneumoniae*, we carried out all experiments in GM17 medium, which is a commonly used medium for *L. lactis*. The growth rate and maximal OD_{600} of strains D39, R6 and TIGR4 were higher in GM17 than in THY or BHI (data not shown). Also, nisin-induced expression of *glnA* in THY seemed to be lower than in GM17. As stability and solubility of nisin are strongly diminished above pH 7 (268), we speculated that this difference was due to the higher pH of THY, which is 7.8 *versus* 6.9 for GM17. To test this hypothesis, nisin induction was performed in GM17 medium with various pH values. In GM17 with a pH higher than 7.5 the level of induction was strongly decreased, while at lower pH values the induction level was comparable to that obtained in standard GM17. As expected, lowering the pH of THY to 7.5 or below led to enhanced induction levels (data not shown). Thus, adjustment of the pH of the growth medium could be a good way to optimize NICE in *S. pneumoniae*.

Complementation of a *glnA* deletion mutant with NICE in chemically defined medium

Previously, complementation in *S. pneumoniae* has been accomplished with the fucose-inducible promoter (226). To validate the improved NICE system, we tested whether *glnA*, expressed under control of the nisin-inducible *nisA* promoter in *S. pneumoniae* D39*nisRK*, could complement a chromosomal deletion in *glnA*. In order to do so, we first needed a CDM to be able to ensure that the *glnA* mutant was auxotrophic for glutamine.

Although chemically defined media for *S. pneumoniae* have been described (4,330,341), they are not commonly used, which could be due to their complexity. We adapted a CDM for *L. lactis* (255), by supplementing it with pyruvate, adenine, uracil, choline chloride, aspartate and cysteine. This CDM is easy to prepare and supported growth of three *S. pneumoniae* strains tested, namely R6, D39 and TIGR4, to a level comparable to that in complex media, such as THY and GM17 (data not shown).

The *glnA* mutant grew well in CDM containing glutamine, but, unlike the wild-type, did not grow in CDM with glutamate instead of glutamine (Fig. 2A). *In trans* expression of *glnA* from the nisin-inducible promoter restored growth in CDM with glutamate instead of glutamine to the level of that in CDM containing glutamine (Fig. 2B), demonstrating the suitability of the NICE system we describe here for complementation experiments in *S. pneumoniae*.

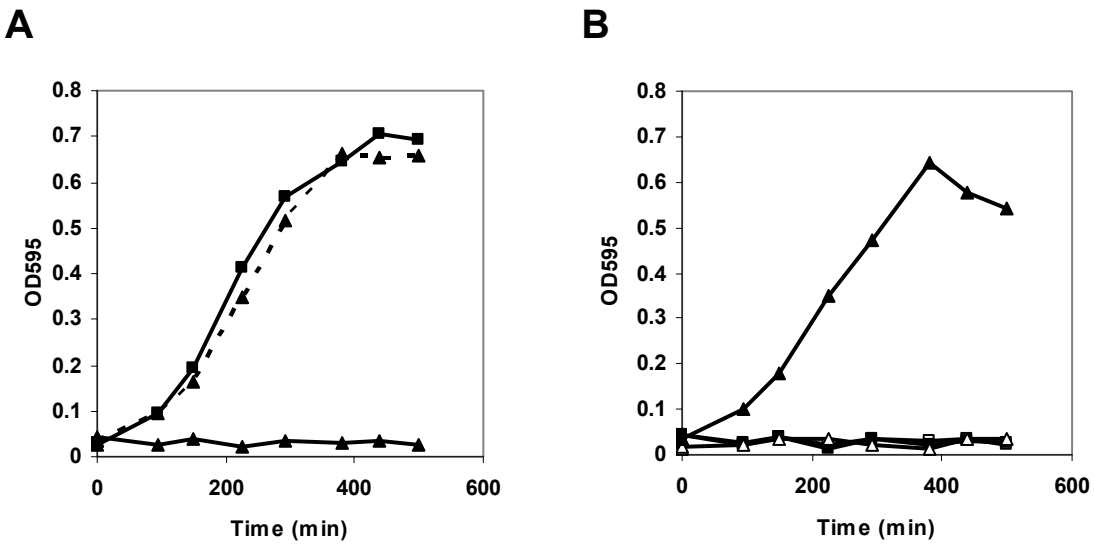

Figure 2. Complementation of the *glnA* deletion in *S. pneumoniae* D39*nisRK*. (A) Growth of strains D39*nisRK* (squares) and TK100 (D39*nisRK* Δ*glnA*;spec) (triangles) in CDM containing 0.4 mg/ml glutamate (solid lines), and growth of TK100 in CDM containing 0.4 mg/ml glutamine (dashed line). (B) Growth of strain TK100 harbouring either plasmid pNG8048E (squares) or pTK6 (triangles) in CDM containing 0.4 mg/ml glutamate and no glutamine. Nisin was added at a concentration of 0 (open symbols) or 2 ng/ml (black symbols).

Taken together, we have adapted the NICE system for *S. pneumoniae* by integrating the *nisRK* genes into the *bgaA* locus, which allows *in trans* nisin-inducible expression of, in principle, every gene of *S. pneumoniae* and solves the limitations observed in earlier studies. In addition, because of the inactivation of endogenous β-galactosidase activity, the strain can be employed for promoter-expression studies using the *E. coli lacZ* gene as a reporter, either or not in combination with nisin-controlled expression of a protein of interest.

Plasmid-based *lacZ* expression system for studying promoter function in *S. pneumoniae*

A plasmid-based GFP reporter system has been applied in *S. pneumoniae* (22,207) but *lacZ* reporter systems described to date are based on integrative plasmids (62,253). The efficiency of integration into the chromosome via insertion-duplication, however, strongly depends on the length of the targeting fragment, which could give problems when a small insert is required (185). More importantly, an integration plasmid is less suitable for the detailed dissection of promoter function, for example by point mutations or deletions.

We developed a plasmid-based *lacZ* reporter system using the lactococcal pWV01 replicon (189). This replicon has been exploited to construct various molecular tools in *L.*

lactis and it functions in both Gram-positive and Gram-negative bacteria (165). Replication of pORI vectors, which are derivatives of pWV01, is dependent on an *in trans* copy of the pWV01 replication initiation gene *repA* (189).

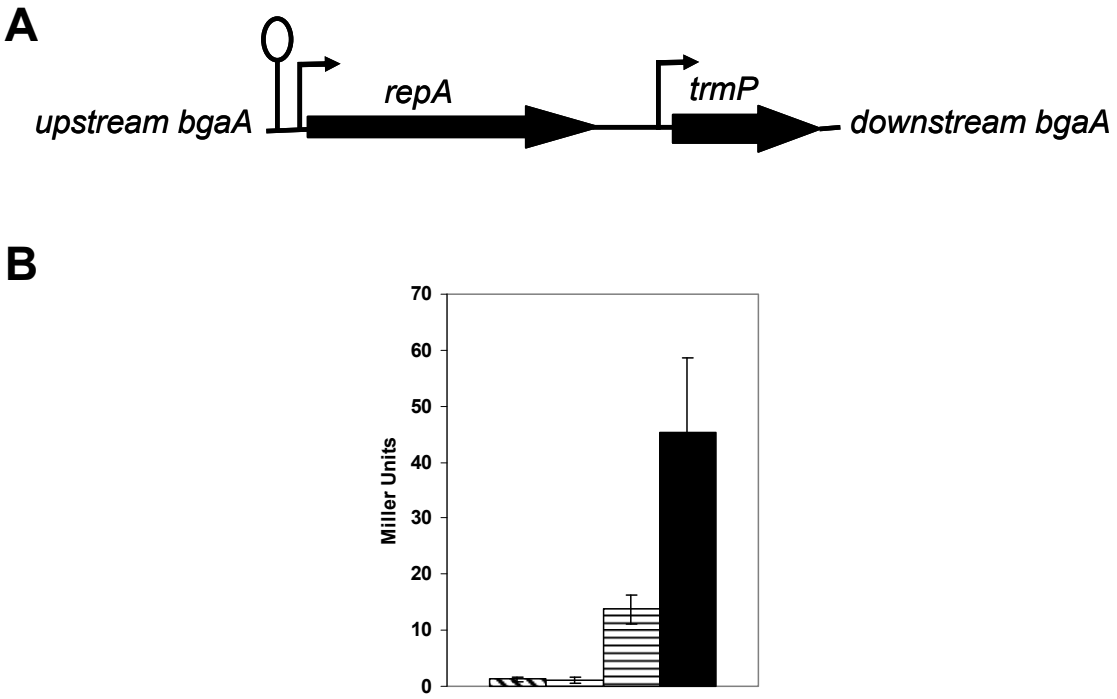

Figure 3. Plasmid-based *lacZ* expression system in *S. pneumoniae*. (A) Schematic representation of the *repA* gene integrated in the chromosome at the *bgaA* locus of *S. pneumoniae* D39. The full-length *bgaA* gene was removed from the chromosome. *trmp*: trimethoprim-resistance marker. Stem-loop indicates terminator structure (predicted ΔG° -20.8 kcal/mole). Right-pointing arrows indicate promoters. (B) β-galactosidase activity of D39*nisRK* (diagonal hatching), D39*repA* (white bar), D39*nisRK* carrying pJB2 (pORI13::P*cpsA*) integrated via single cross-over in the promoter region of *cpsA* (horizontal hatching), and D39*repA* harbouring pJB2 as a plasmid (black bar). Error bars show standard deviation from three independent experiments.

To maintain the pORI vectors in *S. pneumoniae*, strain D39*repA* was constructed, which carries the *repA* gene, driven by a constitutive promoter, in the *bgaA* locus (Fig. 3a). Like strain D39*nisRK*, D39*repA* does not display endogenous β-galactosidase activity (Fig. 3b). Subsequently, we tested whether the RepA-dependent plasmid pORI13 (277), which contains a promoter-less *E. coli lacZ* for transcriptional fusions preceded by a lactococcal ribosome binding site, a multiple cloning site and stop codons in all three reading frames, could be employed as an integrative and replicative *lacZ* reporter vector in *S. pneumoniae* strains D39*nisRK* and D39*repA*, respectively. To this end, the promoter of *cpsA*, the first gene of the locus encoding enzymes necessary for capsule production, was cloned upstream of the *lacZ* of pORI13. The resulting construct was either integrated into the chromosome of strain D39*nisRK* or introduced into strain D39*repA*. The plasmid could be isolated from D39*repA* but not from D39*nisRK* (data not shown). Moreover, D39*repA* could be transformed easily with empty pORI13, while wild-type D39 could not (data not shown), showing that pORI13 is maintained as an autonomous plasmid in D39*repA*. The *cpsA* promoter is active in GM17 medium and *lacZ* expression is approximately threefold higher in strain D39*repA* than in strain D39*nisRK* (Fig. 3b), indicating that pORI13 is present as a low-copy plasmid in the former, giving β-galactosidase activity close to a chromosomal single-copy situation. Likewise, TIGR4 and R6 derivatives harbouring *repA* in the *bgaA* locus were able to replicate pORI13 (data not shown).

Thus, this system enables the use of the *lacZ* expression plasmid pORI13 both for integration and as a low-copy number plasmid, allowing detailed investigation of promoter function. Another advantage of having a $RepA^+$ derivative of *S. pneumoniae* is that there is a range of different pORI vectors, with various antibiotic resistance genes and extensive multiple-cloning sites (187,188), which can be maintained as replicative plasmids in D39*repA* as well (data not shown). In addition, pORI13 can be combined with the NICE system described above, since plasmid pNG8048E, containing the nisin-inducible promoter, provides the pWV01 *repA* gene required for the replication of pORI13 (data not shown).

Construction of unmarked chromosomal mutations with pORI280 in *S. pneumoniae*

Generally, mutant *S. pneumoniae* strains are constructed by substitution of a target gene with an antibiotic resistance gene via a double cross-over event. This method has several disadvantages. First, it could cause polar effects on the expression of downstream genes, especially when the target gene is one of the first genes in an operon. Second, the introduction of point mutations or small in-frame deletions in a wild-type gene on the chromosome is not possible. Third, construction of strains containing multiple mutations could be hampered by the lack of sufficient antibiotic markers. Introduction of unmarked mutations into the chromosome would circumvent these drawbacks.

Previously, *rpsL* has successfully been applied as a counter-selectable marker in *S. pneumoniae* to construct unmarked mutations (310). However, this system requires the presence of a mutation in the wild-type chromosomal copy of *rpsL*. Furthermore, the mutated *rpsL* allele spontaneously converts with low frequency to wild-type, giving rise to false positives (310). Unmarked mutants have also been constructed in *S. pneumoniae* by overlap-extension PCR mutagenesis (305) and by direct transformation with a PCR fragment containing the desired mutation (134). With these methods selection for mutant clones is done by checking for antibiotic-sensitivity and colony PCR.

We investigated whether the lactococcal plasmid pORI280, which contains a constitutively expressed *lacZ* and an erythromycin-resistance gene, can be used for the introduction of unmarked mutations in *S. pneumoniae*. pORI280 was originally developed to generate unmarked mutations in the chromosome of *L. lactis* independent of the genotype of the host and it allows screening of possible mutant clones on plates by visual blue/white selection (187). We used gene *spr0443* (*glnR*) to test the system in *S. pneumoniae*. To this end, chromosomal fragments of 800 bp surrounding *glnR* were inserted into pORI280 and the construct (pTK7) was used to transform *S. pneumoniae* D39 with selection for erythromycin resistance (see Fig. 4 for a schematic representation of the procedure). As replication of pORI280 is dependent on RepA, the transformation leads to single cross-over integration of the construct into the chromosome. After growing several erythromycin-resistant, LacZ-positive integrants as separate cultures for 30-50 generations (culturing 2 to 4 times until stationary phase) without antibiotic selection, cells were plated on X-gal medium to screen for clones that had lost the plasmid as the result of a second recombination event. Of the colonies, 0.5% were both white and erythromycin sensitive, indicating excision of the plasmid from the chromosome. Of these white, erythromycin-sensitive colonies 80% contained the desired mutation, as verified by PCR, Southern blotting and nucleotide sequencing (data not shown). In the same way, we successfully introduced two point mutations in the chromosomal copy of *glnR* of *S. pneumoniae* D39. Both mutant strains will be described in detail elsewhere. An advantage of the use of pORI280 compared to the methods mentioned earlier is that possible mutants can be easily selected on the basis of their white colour on plates with X-gal. Thus, this system provides an efficient way to obtain unlabelled mutants in *S. pneumoniae*.

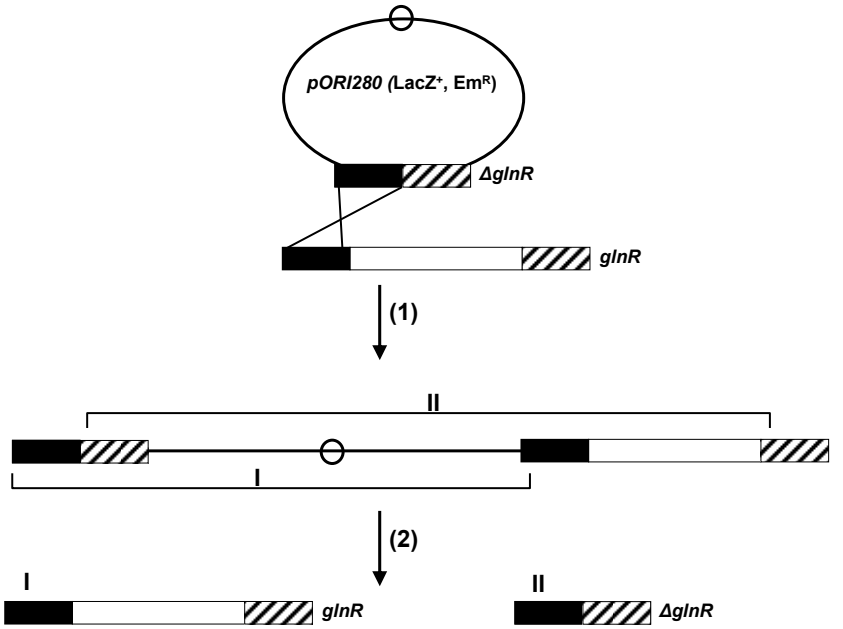

Figure 4. Schematic representation of the use of pORI280 for the construction of unmarked deletions in the chromosome of *S. pneumoniae*. pORI280 containing the left (black rectangle) and right (hatched rectangle) flanking regions of *glnR* is integrated into the chromosome via single cross-over (1); small circle, origin of replication of pWV01. For simplicity only integration via one flanking region is depicted. $LacZ^+$, erythromycin-resistant integrants are grown without selection to allow the excision of pORI280 in a second recombination event through either I or II (2). This results in white, erythromycin-sensitive clones that have either reverted to the wild-type (I) or contain the deleted *glnR* (II).

Conclusions

In this paper, we report on the successful development and employment of important molecular tools for the study of gene function and gene expression in *S. pneumoniae*. These tools were adapted from the *L. lactis* toolbox and complement existing genetic methods. A general benefit is that all cloning steps with *S. pneumoniae* DNA can be performed in *L. lactis*, which in our hands gives fewer problems than *E. coli*. Taken together, the tools offer new possibilities for molecular research on *S. pneumoniae* and will help to unravel the molecular mechanisms that underlie infection by and the pathogenesis of this important human pathogen.

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